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Principal Investigator Organization and Address:	The University of Texas Southwestern Medical Center Dallas, TX 75390
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have shown that the synaptic receptor protein known as EphB1 is a central player in nerve injury-induced neuropathic pain and the related pain symptoms associated with the withdrawal from opioid/morphine addiction. Our hypothesis is that postsynaptic EphB1 participates in pain through the ability of its extracellular domain to form protein-protein interactions with its presynaptic ligand, ephrin-B2, and the NR1 subunit of the postsynaptic NMDA receptor to inappropriately strengthen the synapses in the spinal cord that transmit pain signals into the brain. Our project is to carry out high-throughput screens (HTS) to identify small molecular weight drug-like compounds from a >200,000 complex library that antagonize EphB1 protein-protein interactions. While we originally set out to target the EphB1:NR1 protein-protein interaction, we changed directions in Year 2 due to technical difficulties and are now focusing on the interaction of EphB1 with ephrin-B2. We have made good progress in developing HTS for antagonists that disrupt the EphB1:ephrin-B2 interaction and already have a small number of potential lead compounds from the initial pilot test screen of an 8,000 compound subset of the main library.

15. SUBJECT TERMS

Chronic neuropathic pain, opioid addiction, synaptic plasticity, EphB1 receptor, ephrin-B2, NMDA receptor, drug discovery

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1. **INTRODUCTION:**

Damage to pain sensing peripheral nerves following traumatic injury or other insult, such as diabetic neuropathy or bone cancer growth, strongly elevates the expression of presynaptic ephrin-B2 ligand in the nociceptive peripheral nerve fibers and postsynaptic EphB1 receptor in the dorsal horn neurons in the spinal cord. This elevated expression leads to enhanced formation of protein-protein interactions between presynaptic ephrin-B2 and postsynaptic EphB1 which leads to greater interactions between EphB1 and the NR1 subunit of the NMDA receptor in postsynaptic structures of spinal dorsal horn neurons and this drives an increase in long-term potentiation (LTP) of these synapses. The increased LTP triggers enhanced transmission of pain impulses that project into the brain, leading to classic neuropathic pain states. Similar mechanisms likely explain the severe withdrawal symptoms from the highly addictive opioid family of drugs (e.g. morphine, hydrocodone, and heroin). Whether due to nerve damage and/or withdrawal from opioid-based drugs, neuropathic pain is a serious problem faced by active military personnel, veterans of past service, as well as the general civilian population.

As described in the submitted application, our plan is to carry out high-throughput screens (HTS) to identify small molecular weight drug-like compounds from a >200,000 complex library that antagonize protein-protein interactions made with the postsynaptic receptor EphB1. While we originally set out to target the EphB1:NR1 protein-protein interaction, we changed directions in Year 2 due to technical difficulties described in recent Quarterly Progress Reports and are now focusing on the interaction of EphB1 with its presynaptic ligand, ephrin-B2. We have made good progress in developing HTS assays for antagonists that disrupt the EphB1:ephrin-B2 interaction and already have a small number of potential lead compounds from the initial successful pilot test screen of an 8,000 compound subset of the main library. If successful, we anticipate discovering a new class of 'smart' drugs that will be much more effective at preventing or reversing chronic pain and to help us deal with the spiking increases in addiction to opioids which has lead to large increases in overdoses and deaths.

2. **KEYWORDS:**

Nerve injury pain, chronic pain, neuropathic pain, opioid addiction, ephrin-B2 ligand, EphB1 receptor, NMDA receptor NR1 subunit, presynapse, postsynapse, LTP, protein-protein interaction, antagonist, AlphaScreen assay, high throughput screen (HTS), drug discovery

3. **ACCOMPLISHMENTS:**
   - **What are the major goals of the project?**

Because of technical difficulties expressing and purifying the protein subdomains of EphB1 (its fibronectin type 3, FN3, domains) needed to probe the EphB1:NR1 protein-protein interaction, we changed directions in the past six months to focus attention on the protein-protein interaction of EphB1 with its partner ligand, ephrin-B2. Previous studies have shown that ephrin-B2 is the presynaptic ligand expressed on the nociceptive nerve fibers that interact with the postsynaptic EphB1 receptor expressed on the dorsal horn neurons which together form the signaling complex important for EphB1 interactions with the NR1 subunit of the NMDA receptor to drive synaptic plasticity (LTP) in the dorsal horn neurons which then signal up into the brain neuropathic pain impulses. We

therefore set out to develop a HTS assay that will allow us to probe the EphB1:ephrin-B2 protein-protein interaction and screen against a large >200,000 complex library of small drug-like chemicals for antagonists that will disrupt this interaction.

- **What was accomplished under these goals?**

Strong progress has been made since we changed directions. We have developed a very robust AlphaScreen chemiluminescent assay that probes the EphB1:ephrin-B2 protein-protein interaction in 384 well plates. We have run a test/pilot HTS of this EphB1:ephrin-B2 AlphaScreen assay using 28 x 384 well plates to screen an 8,000 complex subset of the main 200,000 complex chemical library. This resulted in recovery of a small number of potential hit compounds, 22, that reduced the chemiluminescent signal >10% at a concentration of 5 uM compound, with 6 of these compounds reducing over 25% (and one very strong compound showed 71% reduction in signal).

- **What opportunities for training and professional development has the project provided?**

Dr. Henkemeyer provides day-to-day, one-on-one guidance and training of all individuals working on the project, including Melody Karsi, lab manager Frances Sprouse, and Dr. Asim Bepari. UT Southwestern also provides a large and diverse faculty and seminars for advanced professional development.

- **How were the results disseminated to communities of interest?**

As we are just in the beginning stages of screening the library for compounds, we have not publically disseminated any of our data.

- **What do you plan to do during the next reporting period to accomplish the goals?**

We are presently retesting the above mentioned initial 22 hit compounds using the Alpha assay and other biochemical/cellular assays to confirm their ability to antagonize the EphB1:ephrin-B2 interaction. We are also gearing up to begin screening the remaining compounds contained in the full 200,000 compound library.

4. **IMPACT:**
   - **What was the impact on the development of the principal discipline(s) of the project?**

The major impact of our studies to date is that we have developed a very robust chemiluminescent Alpha assay that allows us to measure with a high degree of well-to-well consistency in 384 well plates the EphB1:ephrin-B2 protein-protein interaction. This give us the capability to advance forward in our planned HTS experiments to identify compounds that modulate the presynaptic-postsynaptic interactions of these important synaptic players involved in the formation of neuropathis pain states.

- **What was the impact on other disciplines?**

While our studies are still in their early stages, I believe our work will impact general fields of receptor biology and cell-cell signaling. Moreover, as we are now targeting the EphB1:ephrin-B2 receptor:ligand interaction, potential hit compounds we discover may have more wide-spread utility outside of the nervous system and could thus impact other fields where these molecules have functions, such as in vascular system development and remodeling (e.g. tumor angiogenesis).

What was the impact on technology transfer?

While our studies are still in their early stages, we certainly hope we are able to identify strong antagonists of the EphB1:ephrin-B2 protein-protein interaction. If we are indeed successful, there will be great potential to impact technology transfer as we would have our hands on a new class of drug-like compounds that could be further developed into actual new drugs to treat and/or prevent chronic pain conditions caused by nerve injury and/or withdrawal from opioid abuse.

- **What was the impact on society beyond science and technology?**

As stated above, our high-risk research has the potential to product a high-impact result, new drugs to combat pain and addiction.

5. **CHANGES/PROBLEMS:**
   - **Changes in approach and reasons for change**

Upon binding to their cognate presynaptic ephrin-B ligands, the postsynaptic EphB receptors bind *in cis* to the extracellular segment of the NR1 subunit of the NMDA receptor complex. This tri-molecular interaction is important for the synaptic localization and activation of the NMDA receptor ion channel which functions to induce long-term potentiation (LTP). The interaction of NR1 with EphB receptors is thought to be mediated by the two adjacent FN3 domains (termed a and b) on the ectodomain of the EphB molecule. The EphB1 receptor interaction with the NMDA receptor is particularly important as our previous studies and those of others have shown chronic neuropathic pain caused by nerve damage and withdrawal symptoms associated with addiction to opioids involves presynaptic ephrin-B2 ligand binding to postsynaptic EphB1 which induces *in* cis associations with the postsynaptic NR1 subunit of the NMDA receptor ion channel in the spinal cord dorsal horn neurons and this tri-molecular interaction leads to LTP involved in neuropathic pain states. The original plan was to express and purify large amounts of the EphB1 FN3a and FN3b domains and the NR1 ectodomain and to use these proteins in high throughput protein-protein interactions screens to identify small molecular weight drug-like compounds that would disrupt the FN3:NR1 interaction and ultimately be useful in the treatment of neuropathic pain and opioid addiction. As described extensively in previous reports, we have tried many different methods to express and purify the extracellular FN3 domains of EphB1 and EphB2 receptors and the extracellular segment of the NR1 subunit, and worked hard to demonstrate robust and reproducible protein-protein interactions between the FN3 domains and the NR1 ectodomain. These interaction assays were needed to verify that our purified proteins are properly folded into their native 3 dimensional structures and capable of high-affinity interactions needed for the high-throughput protein-protein interaction assays.

Unfortunately, neither bacterial cell-expressed or mammalian cell-expressed proteins have provided satisfactory yields of protein or consistent protein binding results and we had not been able to develop the necessary reagents and methods to carry out the desired high-throughput screens. To overcome this difficulty, we have devised an alternative approach, developing a protein-protein interaction assay that measures the interaction of EphB1 receptor with its cognate ephrin-B ligands. As the ephrin-B:EphB1 interaction is needed for the EphB1 receptor to interact with the NR1 subunit, we reason that if we can identify small drug-like compounds that disrup the ability of the EphB1 receptor to bind its ephrin-B ligands, these too will have utility as potential drugs to treat pain and addiction. Moreover, using commercially available full ectodomains for the EphB1 protein and ephrin-B ligands (we can use ephrin-B1, ephrin-B2, or ephrin-B3) we have very exciting new data that shows we can measure extremely robust interactions of EphB1:ephrin-B in the AlphaScreen assay. As the ligand ephrin-B2 is specifically implicated in pain (Zhao et al. - Nociceptor-expressed ephrin-B2 regulates inflammatory and neuropathic pain. *Molecular Pain* 2010, 6:77), we focused on scaling up the EphB1:ephrin-B2 Alpha assay to conduct high-throughput screens.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

We have worked through many challenges and with the new approach I am now confident we can move forward without delay in screening of drug compound libraries.

- **Changes that had a significant impact on expenditures**

None, resources have been conserved as we worked our way on refining our HTS assays.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

The original grant contained a small subcontract ($50,000 direct) that was to start in Year 3 and was to conduct preliminary pain tests of the most promising lead compound on an animal model (mice). This work was to be conducted by collaborator Xuejun Song at Parker University. Unfortunately, Parker officials informed us a few months ago that they no longer wanted to support or participate in the research. Although Xuejun and I are not happy about this decision, in reality given our difficulties in developing HTS assays, we do not really stand ready to move forward with animal studies anyway. We will definitely be able to put these freed up funds to good use in Year 3 as we scale up and screen the full >200,000 complex drug library with our newly developed AlphaScreen assay.

- **Significant changes in use or care of human subjects**

N/A

- **Significant changes in use or care of vertebrate animals.**

See above

- **Significant changes in use of biohazards and/or select agents**

N/A

6. **PRODUCTS:**
   - **Publications, conference papers, and presentations**

Nothing to report.

   - **Website(s) or other Internet site(s)**

Nothing to report.

   - **Technologies or techniques**

See above.

   - **Inventions, patent applications, and/or licenses**

Nothing to report.

   - **Other Products**

Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:**
   - **What individuals have worked on the project?**

Name:	Mark Henkemeyer
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Worked on all aspects of project.
Funding Support:	
Name:	Frances Sprouse
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Lab manager, assisted on all aspects of project.
Funding Support:	
Name:	Melody Karsi

Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Expressed and purified proteins from bacterial cells, conducted biochemical protein-protein interactions assays experiments on purified proteins, develop Alpha assays, conduct HTS screens.
Funding Support:	
Name:	Asim Bepari
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Expressed and purified proteins from mammalian cells, conducted biochemical protein-protein interactions assays, and worked on cell-based experiments to investigate ephrin-B:EphB1:NR1 protein-protein interactions in mammalian cells.
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

- **What other organizations were involved as partners?**

Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS:**

COLLABORATIVE AWARDS:

N/A

- **QUAD CHART:**

See attached

9. **APPENDICES:**

N/A

EphB1 as a Novel Drug Target to Combat Pain and Addiction

1115013

Clinical & Rehabilitative Medicine

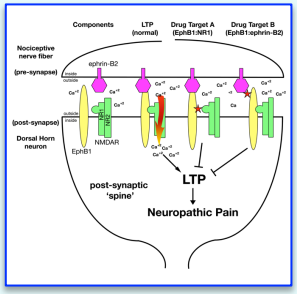
PI: Dr. Mark Henkemeyer **Org:** University of Texas, Southwestern Medical Center **Award Amount:** $1,385,682

Study/Product Aim(s)

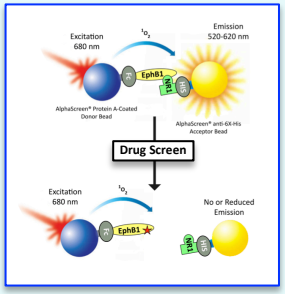
- Aim *1* (*revised*): To screen a library of small drug-like compounds to identify those that antagonize the EphB1:ephrin-B2 protein-protein interaction.
- Aim 2 (*revised*): To synthesize and evaluate EphB1-binding peptoids for ability to antagonize the EphB1:ephrin-B2 protein-protein interaction.

Approach

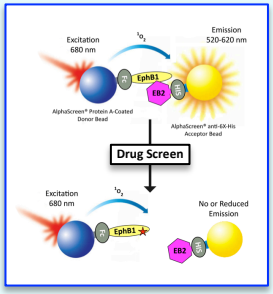
A sensitive AlphaScreen chemiluminescent assay for use on 384 well plates has been developed to probe the EphB1:ephrin-B2 interaction in high-throughput. This assay will be used to screen a library of >200,000 small drug-like chemicals to identify compounds that disrupt the interaction. It will also be used to characterize the antagonistic activities of peptoid compounds derived/converted from known peptide inhibitors of EphB.



Ephrin-B2:EphB1 interaction recruits and activates the NMDA receptor to the post-synapse



Drug Target A EphB1:NR1 interaction



Drug Target B EphB1:ephrin-B2 interaction

Pre-synaptic ephrin-B2 on nociceptive fibers binds post-synaptic EphB1 on dorsal horn neurons and this leads to *in cis* binding of the NR1 subunit and activation of the NMDA receptor, resulting in synaptic plasticity (LTP) implicated in neuropathic pain. Original plans were to develop assays for the EphB1:NR1 interaction (Drug Target A) and revised plan is to probe the EphB1:ephrin-B2 interaction (Drug Target B).

Accomplishments: We have developed a robust EphB1-EphrinB protein-protein interaction assay and have validated its use for HTS screens by conducting an 8K subscreen, resulting in a small number of potential lead hit compounds.

Timeline and Cost

Activities CY	14	15	16	17
Develop Alpha/Peptoid screens				
Execute Alpha/Peptoid screens				
Validate antagonistic compounds				
Estimated Budget ($K)	**$435K**	**$434K**	**$517K**	**$000**

Updated: (UTSW, September 30, 2016)

Goals/Milestones(*revised*)

CY14-15 Goals – Develop protein reagents and interaction assays

☐Clone vectors to make EphB1/FN3 and NR1/NTD domains

☐Purify large amounts of EphB1 and NR1 proteins from bacteria

☐Establish EphB1-NR1 AlphaScreen assay and other assays

CY15-16 Goals – Develop interaction assays and initiate HTS screens

☐Optimize protein-protein HTS interaction assays

☐Begin HTS screens

CY16-17 Goals –HTS and validation

☐Complete HTS of 200,000 complex library

☐Conduct biochemical and cell-based tests of promising lead hits

☐**Comments/Challenges/Issues/Concerns**

- We have conserved funds as we worked to overcome earlier challenges and revised our approaches to allow HTS to proceed.

Budget Expenditure to Date

Projected Expenditure: $108,718 (Y2Q4) / $869,744 (Y1Q1-Y2Q4)

Actual Expenditure: $124,853 (Y2Q4) / $825,217 (Y1Q1-Y2Q4)